Neuropeptide Y Modulates Neurotransmitter Release and Ca²⁺ Currents in Rat Sensory Neurons

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Using ¹²⁵I-labeled neuropeptide Y (NPY) and peptide YY (PYY), we demonstrated the existence of specific receptors for these peptides on rat dorsal root ganglion (DRG) cells grown in primary culture. Scatchard analysis of membrane homogenates indicated that the peptides bound to 2 populations of sites, with approximate affinities of 0.08 and 6.5 nM. Only low levels of binding were detected on sympathetic neurons cultured from the same animals or on a variety of neuronal clonal cell lines. The binding of ¹²⁵I-NPY and ¹²⁵I-PYY to DRG cell membranes was considerably reduced by the nonhydrolyzable analog of GTP, Gpp(NH)p. The major effect of Gpp(NH)p was to reduce the number of lower-affinity NPY binding sites without altering the number of high-affinity binding sites. NPY potently inhibited Ca²⁺ currents recorded under voltage clamp in rat DRG cells. Both the transient and sustained portions of the Ca²⁺ current were inhibited. The inhibitory effects of NPY were completely blocked following treatment of the cells with pertussis toxin. Depolarization elicited a large influx of Ca²⁺ into DRG neurons as assessed using fura-2-based microspectrofluorimetry. This influx of Ca²⁺ could be partially inhibited by NPY. Furthermore, NPY effectively inhibited the depolarization-induced release of substance P from DRG cells *in vitro*. Thus, NPY may be an important regulator of sensory neuron function *in vivo*.

Neuropeptide Y (NPY) is a 36 amino acid peptide neurotransmitter (Tatemoto, 1982a) that is very widely distributed in both the central and peripheral nervous systems (Allen et al., 1983; Chronwall et al., 1985). In many instances, NPY is found to be colocalized with other neurotransmitters such as norepinephrine (Chronwall et al., 1984; Ekblad et al., 1984; Everitt et al., 1984; Jarvi et al., 1986; Wang et al., 1987). NPY is only one member of a family of peptides that also includes peptide YY (PYY) and the pancreatic polypeptides (PP), as well as several others (Tatemoto, 1982b; Tatemoto et al., 1982). NPY and PYY show considerable sequence homologies (Tatemoto et al., 1982). Furthermore, their pharmacological effects are also similar, suggesting that they may act at the same receptors (Lundberg and Tatemoto, 1982; Edvinsson, 1985; Saria and Beubler, 1985; Morley et al., 1985; Friel et al., 1986; Serfozo et al., 1986;

Zukowska-Grojec et al., 1986). PYY is found mostly in the glicentin containing endocrine cells of the gastrointestinal mucosa and may have endocrine or paracrine functions (Bottcher et al., 1984). Circulating PYY has been detected, and its concentration changes under various dietary conditions (Pappas et al., 1986). In addition, however, PYY has also been detected in a unique population of neurons in the CNS (Ekman et al., 1986). These neurons are distinct from those that contain NPY and are less widely distributed.

Not only is NPY a very widely distributed neuropeptide, but it can be shown to produce a very large number of biological effects. Following its injection into the brain, for example, NPY produces many behavioral sequelae. One of the most striking of these is an enormous stimulation of feeding (Morley et al., 1985; Stanley et al., 1986). NPY also acts upon many peripheral systems, including a variety of smooth muscle preparations (Wahlestedt et al., 1985; Wahlestedt and Hakanson, 1986; Zukowska-Grojec et al., 1986), the heart (Lundberg et al., 1984), and intestinal epithelia (Friel et al., 1986; Hubel and Renquist, 1986). Interestingly, at several neuroeffector junctions, NPY appears to have both pre- and postsynaptic effects (Wahlestedt et al., 1985; Wahlestedt and Hakanson, 1986).

Although NPY has been shown to inhibit adenylate cyclase in a number of cases, relatively little is known about the molecular basis of its action (Kassis et al., 1987; Westlind-Danielsson et al., 1987). Some studies have identified high-affinity binding sites for NPY in the nervous system (Unden and Bartfai, 1984; Chang et al., 1986; Harfstraind et al., 1986; Martel et al., 1986). However, nothing is known about the cellular transduction mechanisms involved in the production of NPY's neuronal effects. NPY receptors and immunoreactivity have been previously localized in the dorsal horn of the spinal cord (Hökfelt et al., 1981; Allen et al., 1983; Chronwall et al., 1985; Martel et al., 1986). It seemed, therefore, that NPY might act as a modulator of sensory neurotransmission. In the present study, we demonstrate that sensory neurons *in vitro* possess high concentrations of specific NPY receptors and that activation of such receptors leads to the inhibition of voltage-sensitive Ca²⁺ currents and of neurotransmitter release from these cells.

Materials and Methods

Preparation of ¹²⁵I-NPY and ¹²⁵I-PYY

The peptides were mono-iodinated using the chloramine T method. The reaction mixture contained 0.25 M phosphate buffer, 2.8 nmol peptide, 0.5 nmol ¹²⁵I and 36 nmol chloramine T. The reaction was stopped after 1 min with 525 nmol sodium metabisulfite and transferred to a test tube containing 1.0 ml Sephadex QEA-A25 equilibrated with the following buffer: 0.08 M Trizma HCl, 0.08 M NaCl, 0.02 M HCl, and 0.02% BSA, pH 8.6.

The iodinated peptide was extracted from the Sephadex in batch rinses, which were then pooled and purified on an Altex C_{18} ultrasphere ion-pairing column (5 μm particle size, 0.46 × 25 cm). The mobile phase consisted of acetonitrile and an aqueous buffer: 0.10 M phosphoric acid, 0.02 M triethylamine, 0.05 M $NaClO_4$, all brought to pH 3.0 with NaOH. Several mono-iodinated peaks eluted at 34% (vol/vol) ACN and 30% (vol/vol) ACN for ^{125}I-NPY and ^{125}I-PYY, respectively. Peak fractions were pooled and concentrated, then desalted on a Bio-gel P-2 column (75 × 2.5 cm) equilibrated with 0.01 M Tris, 0.1% BSA, and 0.02% sodium azide, pH 7.4. The peak fractions were pooled and concentrated, then stored at −20°C until needed for experiments. For both NPY and PYY, the HPLC peak that offered maximal synthetic yield and specific binding to rat brain synaptosomes was selected as the choice peak and used in all future binding experiments.

Cell culture

Dorsal root ganglion (DRG) cells were cultured from neonatal rats as previously described (Perney et al., 1986).

Binding studies

DRG cells. Cells were grown for 2 weeks on 35 mm plates. The culture media was aspirated and replaced with binding buffer containing 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 20 mM HEPES, 0.3% BSA, 1 mM DTT, 0.1% bacitracin, 100 mg/liter streptomycin sulfate, 1 mg/liter aprotinin, 10 mg/liter trypsin inhibitor, and 1 μM captopril, pH 7.4. Enough buffer was added so that the addition of cold peptide and iodinated label would bring the total volume to 1 ml. Cold peptide was added to dilute the specific activity of the label at higher concentrations. The maximal concentration of label was 100,000 cpm/ml ≈0.05 nM. The binding was initiated by adding iodinated label to the plates, which were then incubated with gentle shaking for 1 hr at 25°C (equilibrium conditions). The binding buffer was aspirated to terminate the reaction. The plates were quickly rinsed with 1 ml ice-cold binding buffer, aspirated, and layered with a final milliliter of buffer. The cells were scraped from the plate with a rubber policeman, transferred to a test tube, and counted for ^{125}I in a gamma counter.

DRG membranes. Cells were grown for 2 weeks on 35 mm plates. The medium was aspirated and replaced with 0.5 ml of ice-cold binding buffer. The cells were scraped from the plate with a rubber policeman. The suspensions from each plate were then pooled and homogenized with a loose Teflon pestle (10–12 strokes). The resultant homogenate was stored on ice until the incubation began.

The incubation microfuge tubes were filled to a volume of 500 μl with buffer, label, and cold peptide. To initiate the reaction, 500 μl of the tissue homogenate was added to each tube, followed by shaking at 25°C for 1 hr (equilibrium conditions; Walker and Miller, 1987). To terminate the reaction, the microfuge tube was centrifuged at 8700 × g for 15 sec. The supernatant was aspirated and the top of the pellet was rinsed with 1 ml ice-cold binding buffer. The tip of the microfuge containing the pellet was cut off, dropped into a 12 × 75 mm tube, and counted for ^{125}I in a gamma counter.

Protein assays were performed using the Biorad method (Bradford reagent).

Binding data analysis

In all binding experiments, the total bound ligand was analyzed with a nonlinear least-squares curve-fitting program (Yamaoka et al., 1981), which interpreted the binding as having both saturable (specific) and nonsaturable (nonspecific) components according to adsorption isotherm models.

Microspectrofluorimetry

Measurements of $[Ca^{2+}]_i$ in single DRG cells were carried out using the dye fura-2 as previously described (Thayer et al., 1986).

Substance P release

The evoked release of substance P from DRG cells was measured by radioimmunoassay as previously described (Perney et al., 1986).

Electrophysiology

DRG neurons cultured from 1-d-old rat pups were voltage-clamped in the whole-cell patch-clamp configuration (Hamill et al., 1981; Marty and Neher, 1983). Ionic conditions optimized isolation of Ca^{2+} currents: K^+ channel blockers tetraethylammonium (TEA$^+$) and cesium (Cs$^+$) replaced, respectively, Na$^+$ on the outside and K$^+$ on the inside of the neurons, no Na$^+$ was present, and pulses were to the Cl$^-$ equilibrium potential. External solutions contained (in mM) 10 $CaCl_2$, 140 TEACl, 1 $MgCl_2$, and 10 glucose. Internal solutions contained (in mM) 10 EGTA, 140 CsCl, 1 $MgCl_2$, and an ATP regenerating system (14 creatine phosphate, 50 U/ml creatine phosphokinase) to retard run-down of Ca^{2+} currents (Forscher and Oxford, 1985). Both solutions were buffered to pH 7.4 with 10 mM HEPES.

Voltage-clamp pulses (350 msec) to 0 mV were given alternately from −40 and −80 mV at 10 sec intervals. The sustained Ca^{2+} current ("L" type) was elicited from the −40 mV holding potential and the transient Ca^{2+} currents ("N" and "T" types) (Nowycky et al., 1985) were additionally elicited from the −80 mV holding potential.

Inhibition of sustained and transient Ca^{2+} currents by various concentrations of NPY was calculated as follows. Total and sustained Ca^{2+} currents were plotted as a function of time and the pre-exposure time course was extrapolated as a single exponential to estimate the expected magnitudes of sustained and transient (total minus sustained) currents at the end of exposure. The percentage reduction of actual sustained and transient currents compared with these expected magnitudes was then calculated.

The patch-clamp amplifier was the Yale Mark V with 1 GΩ headstage. An Indec 11-23 computer system was used to produce voltage-clamp pulses and record data. Calcium currents were filtered at 500 Hz and digitally sampled at 400 μsec intervals. Leakage and capacitative currents were subtracted from current records by modeling the sum of the appropriate number (8 or 4) of currents produced by 10 mV hyperpolarizing pulses from holding potential and subtracting the modeled current from the raw current traces. NPY was applied by bath superfusion from a polyethylene tube positioned approximately 2 mm from the voltage-clamped neurons.

Statistics

Significance levels were determined using the 2-tailed Student's t test.

Table 1. Binding parameters for DRG cells

System	n	$B_{max(1)}$	$K_{d(1)}$	$B_{max(2)}$	$K_{d(2)}$
^{125}I-NPY					
Intact cells	3	307 ± 19	0.62 ± 0.15	—	—
Membranes	3	26 ± 10	0.08 ± 0.03	971 ± 143	6.5 ± 4.0
Membranes + Gpp(NH)p	3	15 ± 10	0.12 ± 0.05	228 ± 130	1.5 ± 0.6
^{125}I-PYY					
Intact cells	1	220	0.43	—	—
Membranes	1	20	0.03	2170	1.2

B_{max} is reported as fmol/mg. K_d is reported as nM. Absence of a detectable binding site is indicated (−). [Gpp(NH)p] = 0.1 mM. Values are reported as means ± SE.
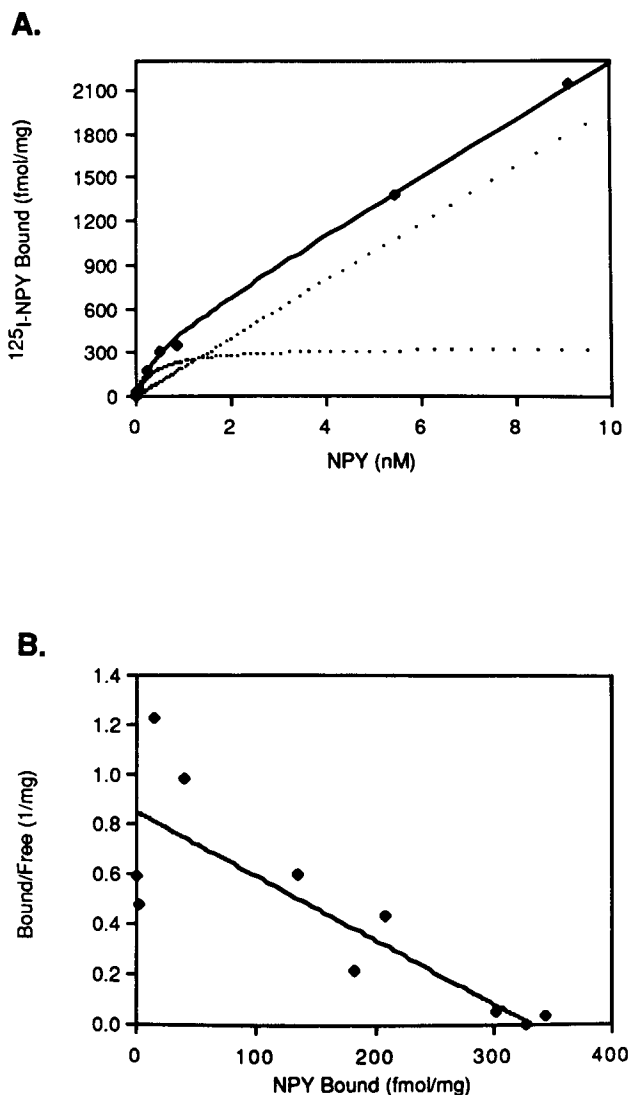
A.

B.

Figure 1. Equilibrium binding of ¹²⁵I-NPY to intact DRG cells (also see Table 1). A representative experiment is shown ($n = 3$). *A*, Direct plot. *Solid line* with data points represents measured total binding; *dotted lines* represent saturable and nonsaturable components as obtained by nonlinear least-squares computer analysis. *B*, Scatchard plot of saturable binding component in *A*. Each point represents binding to an individual DRG culture.

Materials

NPY, PYY, substance P, and Gpp(NH)p were purchased from Sigma (St. Louis). Pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA). ¹²⁵I was purchased from Amersham (Arlington Heights, IL). All other chemicals were reagent grade.

Results

Both ¹²⁵I-NPY and ¹²⁵I-PYY exhibited a high degree of specific binding to intact cultured rat DRG cells (Table 1). Binding curves obtained using intact cells indicated the presence of a single site with a K_d of 0.62 ± 0.15 nM and a B_{max} of 307 ± 19 fmol/mg protein for ¹²⁵I-NPY (Fig. 1, Table 1). A 2-site model did not yield a significantly better fit of the data. Considering the relatively large amount of nonspecific binding inherent in whole-cell binding studies, it is possible that a second site was obscured. However, a 1-site model appears to be the most parsimonious interpretation of the data. The characteristics of the

binding curves changed when membranes isolated from DRG cells were used. Scatchard plots became clearly curvilinear, indicating the presence of more than one kind of binding site (Fig. 2, Table 1). Thus, for both ligands binding studies on isolated membranes revealed the presence of 2 binding sites. For example, studies with ¹²⁵I-NPY indicated the presence of sites with K_d's of 0.08 ± 0.03 and 6.5 ± 4.0 nM.

When DRG cell membranes were treated with Gpp(NH)p, a nonhydrolyzable analog of GTP, the specific binding of ¹²⁵I-NPY and ¹²⁵I-PYY decreased considerably (Figs. 3, 4). Concentrations of Gpp(NH)p as low as 10^{-6} M were effective in this respect. The decrease in binding associated with the effects of Gpp(NH)p appeared to be due to the disappearance of many of the lower-affinity binding sites, so that the binding more closely resembled the whole-cell profile. It is not clear what the basis of this decline in the B_{max} of the lower-affinity sites might be. One possibility is that the sites are transformed into a further form with such a low affinity that it is difficult to detect owing to limitations of the binding assay (i.e., relatively high nonspecific binding in the low-affinity concentration range).

In contrast to DRG neurons, only low levels of binding sites were observed on neurons cultured from the superior cervical ganglia of neonatal rats, on normal or differentiated PC12 cells, or on NG108-15 or F-11 clonal neuronal cell lines (Table 2).

We next investigated the functional consequences of activation of the NPY receptors located on DRG neurons. When DRG cells were depolarized by raising [K⁺]ₒ from 5 to 50 mM, the release of substance P into the bathing medium was stimulated (Perney et al., 1986). This release could be significantly inhibited by 10^{-7} M NPY (Fig. 5). It has been previously demonstrated that several other neurotransmitters can also block the evoked release of substance P from DRG cells, an effect that is associated with the inhibition of DRG Ca²⁺ currents (Mudge et al., 1979; Dunlap and Fischbach, 1981). We therefore examined the effects of NPY on DRG Ca²⁺ currents recorded using the whole-cell patch-clamp method. When cells were held at hyperpolarized potentials (-80 mV), steps to 0 mV evoked a large Ca²⁺ current that exhibited both transient and sustained components. This pattern is similar to that previously observed in chick DRG cells (Nowycky et al., 1985). When cells were held at a relatively depolarized potential (-40 mV), steps to 0 mV evoked Ca²⁺ currents which exhibited little inactivation. It has been shown previously using chick DRG cells that the overall form of the DRG Ca²⁺ current is due to the activation of 3 separate types of voltage-sensitive Ca²⁺ channels (Nowycky et al., 1985). The sustained portion of the current is primarily due to the activation of L channels, whereas the transient portion of the current is due to the activation of N and T channels. These latter channel types are mostly inactivated when cells are held at -40 mV.

10^{-7}M NPY dramatically reduced the magnitude of the DRG Ca²⁺ current. Both phases of the Ca²⁺ current appeared to be reduced. Figure 6 illustrates the effects of NPY on the peak Ca²⁺ current evoked from -80 mV (transient plus sustained) and on the sustained current evoked from -40 mV. Inhibitory effects were observed at NPY concentrations as low as 10^{-10} M and half-maximal effects at about 10^{-9} M (Fig. 7). The Ca²⁺ current recovered following washout of the peptide. The effects produced by NPY were extremely reliable. Thus, the peptide inhibited the Ca²⁺ current in every cell tested.

The binding studies discussed above suggest that NPY receptors in DRG cells are associated with G-proteins. We therefore examined the effects of NPY following treatment of DRG cells
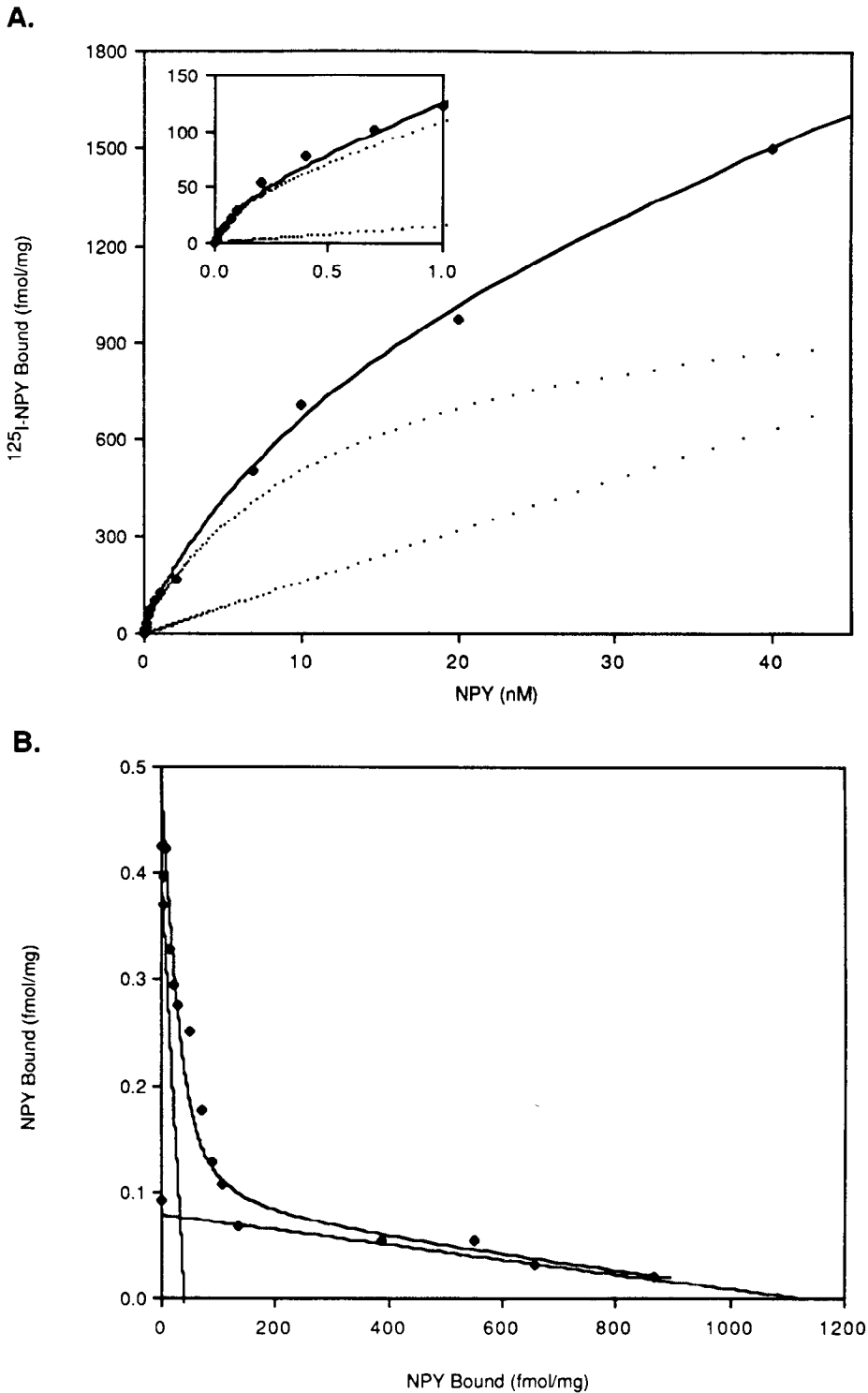
A.

B.

NPY Bound (fmol/mg)

Figure 2. Equilibrium binding of ^{125}I-NPY to DRG membranes. Also see Table 1). A representative experiment is shown ($n = 3$). *A*, Direct plot. *Solid line* with data points represents measured total binding; *dotted lines* represent saturable and nonsaturable components as obtained by nonlinear least-squares computer analysis. *B*, Scatchard plot of saturable binding component in *A*. All points represent triplicate determinations.

with pertussis toxin (Fig. 8). Following such treatment the inhibitory effects of 10^{-7} M NPY were no longer apparent. Pertussis toxin blocked the effects of NPY on all phases of the Ca^{2+} current.

We next examined the effects of NPY on the voltage-dependent uptake of Ca^{2+} into single DRG neurons using fura-2 based microspectrofluorimetry. The resting $[Ca^{2+}]_i$ in DRG neurons was approximately 10^{-7}M. When cells were depolarized by raising $[K^+]_o$ from 5 to 50 mM, $[Ca^{2+}]_i$ rapidly increased. Treatment of cells with 10^{-7}M NPY partially inhibited this depolarization-

induced rise in $[Ca^{2+}]_i$ (Fig. 9). The inhibitory effects of NPY were no longer apparent if cultures were treated with pertussis toxin ($n = 2$).

Discussion

Receptors for NPY and PYY are extremely widely distributed both in the CNS and in the periphery (Chang et al., 1986; Harfstraind et al., 1986; Martel et al., 1986). In studies to be reported elsewhere we have found that both ^{125}I-NPY and ^{125}I-PYY exhibit curvilinear Scatchard plots and and appear to label 2
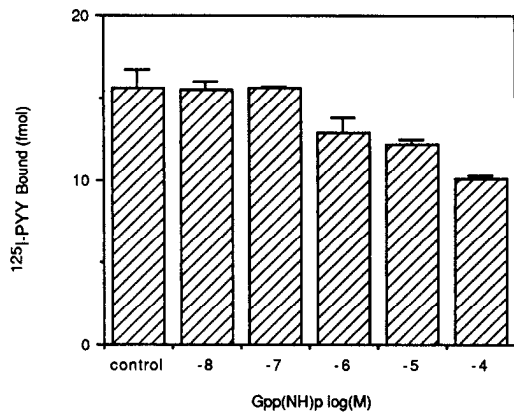
Figure 3. Effect of Gpp(NH)p on ¹²⁵I-PYY binding to DRG membranes. Membranes were incubated at 25°C for 60 min with 0.15 nM ¹²⁵I-PYY. All points represent triplicate determinations.

distinct binding sites in rat brain synaptosomes (Walker and Miller, 1987), a similar situation to that found in DRG membranes. Furthermore, biochemical studies indicate that both ligands can interact with both binding sites. It seems, therefore, that receptors for NPY and PYY may actually be the same molecule(s), biological specificity being derived from the presence or absence of the particular peptide agonist.

One area in which both NPY receptors and immunoreactivity exist in high concentrations is in the dorsal horn of the spinal cord (Hökfelt et al., 1981; Allen et al., 1983; Chronwall et al., 1985). In the present study we have demonstrated that receptors exist on sensory neurons *in vitro*. It is likely, therefore, that much of the binding normally observed in the substantia gelatinosa is on the terminals of incoming sensory neurons. Such
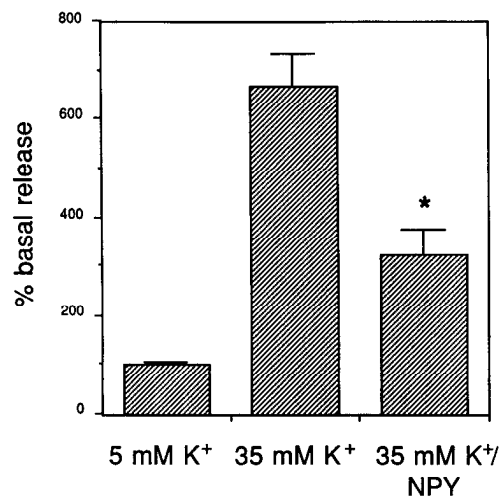

Figure 5. NPY inhibits K⁺ depolarization-induced release of substance P-like immunoreactivity from DRG cultures. Basal levels of release were approximately 4 fmol/ml/100 μg protein. The release evoked by a 5 min depolarization with 35 mM K⁺ was measured in the presence or absence of 10⁻⁷ M NPY. Results are presented as the percentage of K⁺-evoked release in cells not exposed to NPY preincubation. NPY, 10⁻⁷ M, inhibited the K⁺-evoked release by 52% (* $p < 0.001$; $n = 7$).

a result is consistent with the idea that one normal function of NPY might be to modulate sensory information flowing into the CNS. In support of this proposal, we have also demonstrated that NPY can reduce the depolarization-evoked release of substance P from DRG cells *in vitro*. Substance P is usually associated with small unmyelinated polymodal nociceptors (Aronin et al., 1983). Thus, NPY may be involved in the normal control
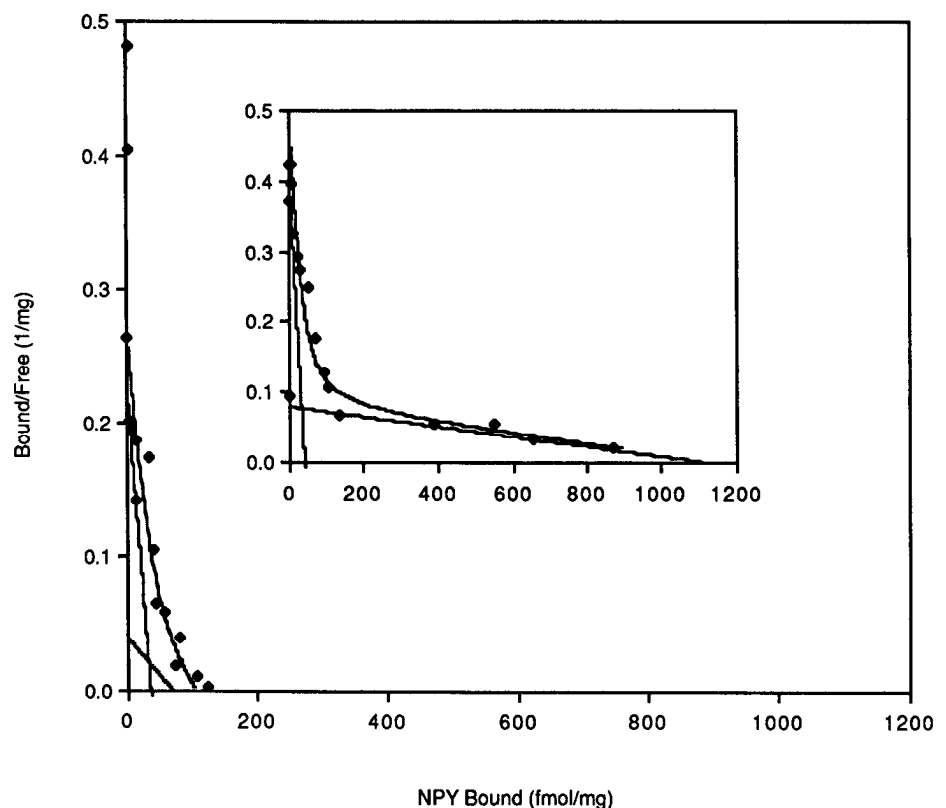

Figure 4. Effect of Gpp(NH)p on ¹²⁵I-NPY binding to DRG membranes (also see Table 1). A representative experiment ($n = 3$). Membranes were incubated at 25°C for 60 min in the presence of 0.1 mM Gpp(NH)p. The saturable binding component is plotted as a Scatchard plot. *Inset,* Saturable binding component in the absence of 0.1 mM Gpp(NH)p. All points represent triplicate determinations.
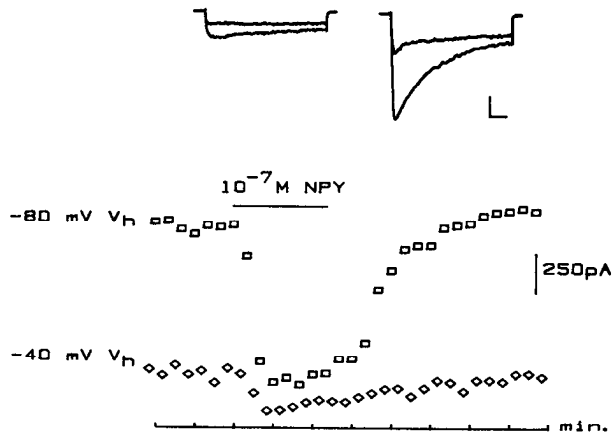
Figure 6. Inhibition of Ca^{2+} currents by 10^{-7} M NPY. A representative experiment is shown ($n = 5$). DRG neurons were voltage-clamped in the whole-cell mode under ionic conditions optimal for isolation of Ca^{2+} currents (see Materials and Methods). Voltage-clamp pulses (350 msec) to 0 mV were alternately given at 10 sec intervals from holding potentials of -40 mV to evoke the sustained Ca^{2+} current (L type) and from -80 mV to evoke the transient Ca^{2+} currents (N and T types) also. The effect of a 3 min exposure to 10^{-7} M NPY (indicated by the *horizontal bar* above the time scale) is plotted as a function of time for total (*squares*) and sustained Ca^{2+} currents (*diamonds*). Traces at the top compare the sustained (*left*) and total (*right*) Ca^{2+} currents at the end of exposure to NPY with currents just prior to exposure. Scale bars for current traces are 250 pA and 50 msec. [See Ewald et al. (1988) for another example.]
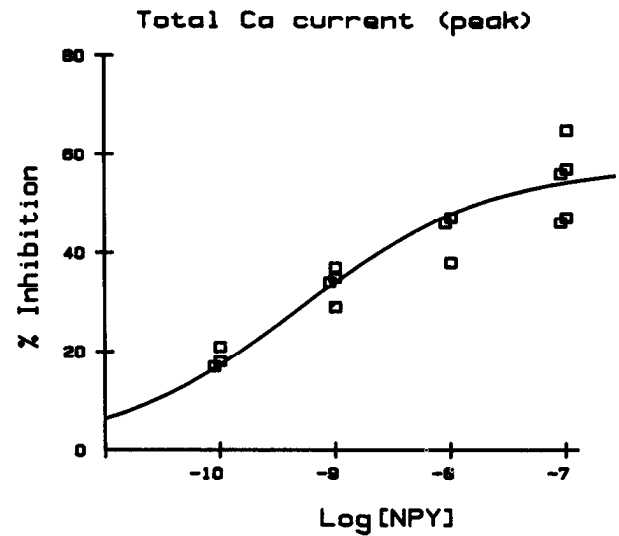
Figure 7. Dose–response curve for inhibition of total Ca^{2+} current by NPY. The time course of the total Ca^{2+} current prior to exposure is extrapolated as a single exponential to the end of exposure. The inhibition produced by a 3 min exposure to various concentrations of NPY is expressed as the percentage reduction relative to the extrapolated baseline. Points were fitted by eye with a sigmoidal curve. The half-maximally effective dose is approximately 1 nM.

of pain and possibly other sensory modalities. NPY receptors appear to be one of a family of neurotransmitter receptors that can be found on sensory neurons and whose activation leads to the inhibition of substance P release. Other members of this group include α_2, GABA-B (Holz et al., 1988) and κ-opioid receptors (Mudge et al., 1979).

A question of considerable interest is how these receptors function at a molecular level and, in particular, how their activation produces inhibition of substance P release. There are several ways in which this could be achieved. For example, a change in K^+ permeability (Kandel and Schwartz, 1982) or in intracellular Ca^{2+} buffering (Boyle et al., 1984) could be involved. However, the most direct method would be by inhibition of the voltage-dependent influx of Ca^{2+} into the nerve terminal. It appears that such a mechanism is widely used in both vertebrate and invertebrate neurons (Miller, 1987). In vertebrate neurons, this phenomenon has, in fact, been most fre-

quently studied in DRG cells (Dunlap and Fischbach, 1981; Werz and MacDonald, 1984; Dolphin et al., 1986). All the different neurotransmitters discussed above that block substance P release also suppress Ca^{2+} currents in DRG cell bodies. If such a mechanism also occurs in the nerve terminal, then it is presumably the basis for the inhibition of substance P release. It should be noted that not all agents that block substance P release from DRG neurons do so by blocking Ca^{2+} currents. Thus, opioids that act on μ- or δ-receptors, for example, appear to act by enhancing one or more DRG K^+ conductances (Miller, 1984).

Table 2. ^{125}I-NPY binding to membranes from various cell types

Cell type	^{125}I-NPY bound (fmol/mg)
Rat dorsal root ganglia	10.95 ± 2.98
Rat sympathetic ganglia	4.45 ± 1.35
HSWP fibroblasts	0.23 ± 0.15
PC12 (rat pheochromocytoma), differentiated with dibutyryl cAMP and nerve growth factor	0.21 ± 0.26
F-11 (rat neuroblastoma × DRG), differentiated with dibutyryl cAMP and nerve growth factor	0.23 ± 0.13

Cell membranes were incubated with 20 pM ^{125}I-NPY for 1 hr at 25°C. Specific binding was determined in the presence of 500 nM unlabeled NPY. In the case of DRG, specific binding was determined from equilibrium curves derived from nonlinear least-squares analysis. All points are reported as means $\pm$ SE ($n = 3$).
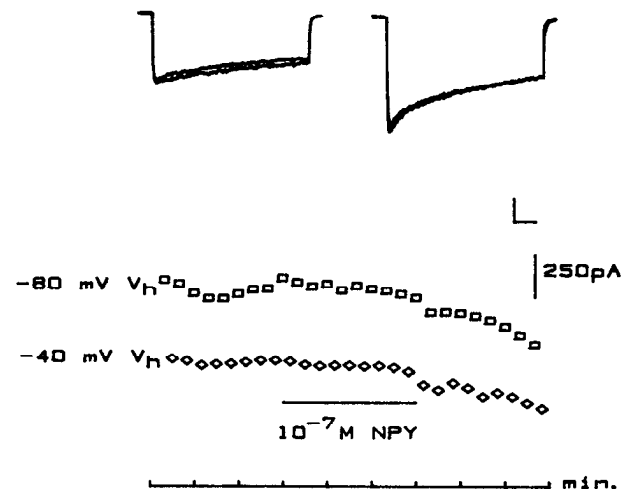
Figure 8. Block of NPY inhibition of DRG Ca^{2+} current by pretreatment with pertussis toxin. DRG neurons were exposed to pertussis toxin in culture media (350 ng/ml) for 16–48 hr before recording Ca^{2+} currents. The data shown here were recorded after 40 hr of pertussis toxin treatment. The experimental protocol and graphic format are identical to that in Figure 6. Inhibition by 10^{-7} M NPY was completely blocked in all of 10 experiments.
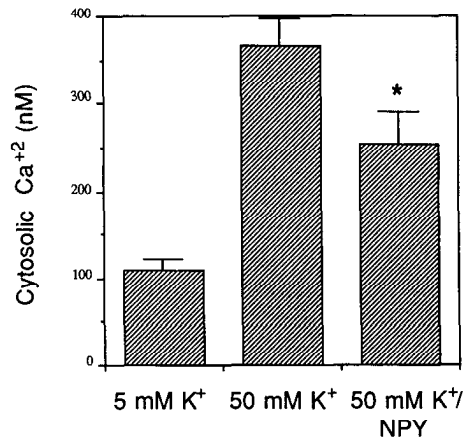
Figure 9. Inhibition of depolarization-induced peak increase in $[Ca^{2+}]_i$ in single DRG neurons by $(10^{-7}$ M NPY) $(*p < 0.025)$. Cells were depolarized by raising $[K^+]_o$ from 5 to 50 mM $(n = 8)$ (Thayer et al., 1987).

Binding studies with DRG membranes revealed the presence of 2 classes of NPY binding sites, as also found in the brain. The significance of this observation in physiological terms is difficult to assess. A reasonable argument can be made, however, that the effects of NPY on DRG Ca^{2+} currents are mediated by the lower-affinity sites. First, the EC_{50} for the effect of NPY on DRG Ca^{2+} currents is similar to the K_d of the low-affinity site or, indeed, to the K_d for the single site observed in whole-cell binding studies (approximately 1 nM). Second, in membrane preparations the properties of the low-affinity site are modulated by Gpp(NH)p, indicating that it is linked to a G-protein. The observation that pertussis toxin blocks the effects of NPY on the DRG Ca^{2+} current further indicates the involvement of a G-protein. With respect to the binding studies using DRG membranes, it may be considered strange that the major effect of Gpp(NH)p is on the B_{max}, rather than the K_d, of the low-affinity site. However, there are several precedents for such an observation, including studies on the labeling of NPY receptors reported by other authors (Unden and Bartfai, 1984; Chang et al., 1985; Nakajima et al., 1986). The fate of these sites is not clear. It is possible that they represent an inositol lipid-linked form of the receptor that can be shed from the membrane under the influence of a Gpp(NH)p activated enzyme such as phospholipase C (Hemperly et al., 1986). Indeed, there are now several examples of proteins that are anchored in the membrane by being covalently linked to inositol phospholipids. More likely, however, is a shift in the state of the receptor to one of very low affinity. Indeed, other studies we have conducted using brain membranes have demonstrated that the effects of Gpp(NH)p are reversible. However, the major point to be made is that DRG cells possess large numbers of NPY binding sites. Some of these appear to be linked to G-proteins. Furthermore, the affinity of such sites is consistent with the effects of NPY on DRG Ca^{2+} currents.

NPY is a very potent inhibitor of DRG Ca^{2+} currents and of the depolarization-induced entry of Ca^{2+} into DRG cells as assessed by fura-2-based microspectrofluorimetry. As with other reported inhibitory modulators of DRG Ca^{2+} currents, its effects are reversed upon treating cells with pertussis toxin (Holz et al., 1986). This indicates the involvement of a G-protein of the G_i or G_o type. NPY appears to inhibit both the sustained and transient portions of the DRG Ca^{2+} current. By analogy

with other results on chick DRG cells, it may be supposed that the sustained portion of the rat DRG current is due primarily to the activation of L channels (Nowycky et al., 1985). This would be reasonable, as we have previously shown that the evoked release of substance P from rat DRG cells is dependent primarily on the activation of L channels. Thus, if NPY can inhibit substance P release, then presumably it should be able to inhibit L currents. However, although such a series of observations would be internally consistent, it cannot be assumed a priori that the various phases of the rat DRG Ca^{2+} current are due to the activation of channels with exactly the same properties as those underlying the chick DRG Ca^{2+} current. Indeed, it is quite clear that the inactivation kinetics of N-type currents do differ considerably in different types of neurons and possibly even in DRG cells from different species (Bean, 1987; Hirning et al., 1987). Thus, the ultimate proof that any particular type of Ca^{2+} channel is being modulated must await studies of this phenomenon at the single-channel level. Until that time, however, the above description of events seems the most reasonable and is at least consistent with other data in the literature.

The molecular mechanisms whereby NPY (or other modulators) block DRG Ca^{2+} currents are not known with any certainty. Some evidence suggests a role for the enzyme protein kinase C (Rane and Dunlap, 1986; Ewald et al., 1988). In the present case, it is noteworthy that the effects of NPY on all phases of the Ca^{2+} current can be reversed by pertussis toxin. If, indeed, the various phases of the current are due to the activation of different populations of Ca^{2+} channels, this suggests that G-proteins of the G_i or G_o type must be involved in the NPY-induced modulation of all these various channel types.

NPY has been shown to modulate the release of neurotransmitters from other neurons as well, including some sympathetic neurons and hippocampal pyramidal cells (Lundberg and Stjarne, 1984; Colmers et al., 1987). It will be interesting to see if these cases also involve the modulation of neuronal Ca^{2+} currents.

References

Allen, Y. S., T. E. Adrian, J. M. Allen, K. Tatemoto, T. J. Crow, S. R. Bloom, and J. M. Polak (1983) Neuropeptide Y distribution in the rat brain. Science 221: 877–879.

Aronin, N., M. DiFiglia, and S. E. Leeman (1983) Substance P. In *Brain Peptides,* D. T. Krieger, M. J. Brownstein, and J. B. Martin, eds., pp. 783–803, Wiley-Interscience, New York.

Bean, B. P. (1987) Norepinephrine changes the voltage dependence of calcium channels in sensory neurones. Biophys. J. 51: 30a.

Bottcher, G., K. Sjolund, E. Ekblad, R. Hakanson, T. W. Schwartz, and F. Sundler (1984) Coexistence of peptide YY and glicentin immunoreactivity in endocrine cells of the gut. Reg. Peptide 8: 261–266.

Boyle, M. B., M. Klein, S. J. Smith, and E. R. Kandel (1984) Serotonin increases intracellular Ca^{2+} transients in voltage-clamped sensory neurons of *Aplysia californica.* Proc. Natl. Acad. Sci. USA 81: 7642–7646.

Chang, R. S. L., V. J. Lotti, T.-B. Chen, D. J. Cerino, and P. J. Kling (1985) Neuropeptide Y binding sites in rat brain labeled with ¹²⁵I-Bolton Hunter NPY: Comparative potencies of various polypeptides on brain NPY binding and biological responses in rat vas deferens. Life Sci. 37: 2111–2122.

Chang, R. S. L., V. J. Lotti, and T.-B. Chen (1986) Increased neuropeptide Y receptor binding in hippocampus and cortex of spontaneously hypertensive (SH) rats compared to normal controls. Neurosci. Lett. 67: 272–275.

Chronwall, B. M., T. N. Chase, and T. L. O'Donohue (1984) Coexistence of neuropeptide Y and somatostatin in rat and human cortical and rat hypothalamic neurons. Neurosci. Lett. 52: 213–217.

Chronwall, B. M., D. A. DiMaggio, V. J. Massari, V. M. Pickel, D. A. Ruggiero, and T. L. O'Donohue (1985) The anatomy of neuropeptide-Y containing neurons in rat brain. Neuroscience 15: 1159–1181.

Colmers, W. F., K. Lukowiak, and Q. J. Pittman (1987) Presynaptic action of neuropeptide Y in area CA1 of the rat hippocampal slice. J. Physiol. (Lond.) *383:* 285–299.

Dolphin, A. C., S. R. Forda, and R. H. Scott (1986) Calcium dependent currents in cultured rat dorsal root ganglion neurones are inhibited by an adenosine analogue. J. Physiol. (Lond.) *373:* 47–61.

Dunlap, K., and G. D. Fischbach (1981) Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurones. J. Physiol. (Lond.) *317:* 519–535.

Edvinsson, L. (1985) Characterization of the contractile effect of neuropeptide Y in the feline cerebral arteries. Acta Physiol. Scand. *125:* 33–41.

Ekblad, E., R. Hakanson, and F. Sundler (1984) VIP and PHI coexist with NPY-like peptides in intramural neurones of the small intestine. Reg. Peptides *10:* 47–58.

Ekman, R., C. Wahlestedt, G. Bottcher, F. Sundler, R. Hakanson, and P. Panula (1986) Peptide YY like immunoreactivity in the central nervous system of the rat. Reg. Peptides *16:* 157–168.

Everitt, B. J., T. Hökfelt, L. Terenius, K. Tatemoto, V. Mutt, and M. Goldstein (1984) Differential coexistence of neuropeptide Y (NPY) like immunoreactivity with catecholamines in the central nervous system of the rat. Neuroscience *11:* 443–462.

Ewald, D. A., H. J. G. Matthies, T. M. Perney, M. W. Walker, and R. J. Miller (1988) The effect of down regulation of protein kinase C on the inhibitory modulation of dorsal root ganglion neuron Ca²⁺ currents by neuropeptide Y. J. Neurosci. *8:* 2447–2451.

Forscher, P., and G. S. Oxford (1985) Modulation of calcium channels by norepinephrine in internally dialyzed avian sensory neurones. J. Gen. Physiol. *85:* 743–763.

Friel, D. A., M. W. Walker, and R. J. Miller (1986) Neuropeptide Y: A powerful modulator of epithelial ion transport. Br. J. Pharmacol. *88:* 425–443.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. Sigworth (1981) Improved patch clamp techniques for high resolution current recording from cells and cell free membrane patches. Pfluegers Arch. *391:* 85–100.

Harfstraind, A., K. Fuxe, L. F. Agnati, F. Benfenati, and M. Goldstein (1986) Receptor autoradiographical evidence for high densities of ¹²⁵I-neuropeptide Y binding sites in nucleus tractus solitarius of the normal male rat. Acta. Physiol. Scand. *128:* 195–200.

Hemperly, J., G. R. Edelman, and B. A. Cunningham (1986) cDNA clones of the neural cell adhesion molecule (N-CAM) lacking a membrane-spanning region consistent with evidence for membrane attachment via a phosphatidylinositol intermediate. Proc. Natl. Acad. Sci. USA *83:* 9822–9826.

Hirning, L. D., A. P. Fox, E. W. McCleskey, B. M. Olivera, S. A. Thayer, R. J. Miller, and R. W. Tsien (1987) Dominant role of N-type calcium channels in K⁺-evoked release of norepinephrine from rat sympathetic neurons. Science *239:* 57–61.

Hökfelt, T, J. M. Lundberg, G. Jansco, and J. Kimmel (1981) Avian pancreatic polypeptide (APP) immunoreactive neurons in the spinal cord and spinal trigeminal nucleus. Peptides *2:* 81–87.

Holz, G. G., S. G. Rane, and K. Dunlap (1986) GTP binding proteins mediate transmitter inhibition of voltage dependent calcium channels. Nature *319:* 670–672.

Holz, G. G., R. M. Kream, and K. Dunlap (1988) Norepinephrine and gamma aminobutyric acid inhibit electrically evoked release of substance P from dorsal root ganglion neurones. J. Neurosci. *8:* 463–471.

Hubel, K. A., and K. S. Renquist (1986) Effect of neuropeptide Y on ion transport by the rabbit ileum. J. Pharmacol. Exp. Ther. *238:* 167–169.

Jarvi, R., P. Helen, M. Pelto-Huikko, and A. Hervonen (1986) Neuropeptide Y (NPY) like immunoreactivity in rat sympathetic neurones and small granule containing cells. Neurosci. Lett. *67:* 223–277.

Kandel, E. R., and J. H. Schwartz (1982) Molecular biology of learning: Modulation of transmitter release. Science *261:* 6637–6639.

Kassis, S., M. Olesmaa, L. Terenius, and P. Fishman (1987) Neuropeptide Y inhibits cardiac adenylate cyclase through a pertussis toxin sensitive G-protein. J. Biol. Chem. *262:* 3429–3431.

Lundberg, J. M., and L. Stjarne (1984) Neuropeptide Y (NPY) depresses the secretion of ³H-noradrenaline and the contractile response evoked by field stimulation in rat vas deferens. Acta Physiol. Scand. *120:* 477–479.

Lundberg, J. M., and K. Tatemoto (1982) Pancreatic polypeptide family (APP, BPP, NPY and PYY) in relation to sympathetic vasoconstriction resistant to α-adrenoceptor blockade. Acta Physiol. Scand. *116:* 393–397.

Lundberg, J. M., X.-Y. Hua, and A. Franco-Cereceda (1984) Effects of neuropeptide Y (NPY) on mechanical activity and neurotransmission in the heart, vas deferens and urinary bladder of the guinea pig. Acta Physiol. Scand. *121:* 325–332.

Martel, J. C., S. St-Pierre, and R. Quirion (1986) Neuropeptide Y receptors in rat brain: Autoradiographic localization. Peptides *7:* 55–60.

Marty, A., and E. Neher (1983) Tight seal whole cell recording. In *Single Channel Recording,* B. Sakmann and E. Neher, eds., pp. 107–122, Plenum, New York.

Miller, R. J. (1984) How do opiates act? Trends Neurosci. *7:* 184–185.

Miller, R. J. (1987) Calcium channels in neurones. In *Structure and Physiology of the Slow Inward Calcium Channel,* D. J. Triggle and J. C. Venter, eds., pp. 161–246, Plenum, New York.

Morley, J. E., A. S. Levine, M. Grace, and J. Kneip (1985) Peptide YY (PYY) is a potent orexigenic agent. Brain Res. *341:* 200–203.

Mudge, A. W., S. F. Leeman, and G. D. Fischbach (1979) Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. Proc. Natl. Acad. Sci. USA *76:* 527–532.

Nakajima, T., Y. Yashima, and K. Nakamura (1986) Quantitative autoradiographic localization of neuropeptide Y receptors in the rat lower brainstem. Brain Res. *386:* 144–150.

Nowycky, M. C., A. P. Fox, and R. W. Tsien (1985) Three types of neuronal calcium channels with different calcium agonist sensitivity. Nature *316:* 440–443.

Pappas, T. W., H. T. Debas, A. M. Chang, and I. L. Taylor (1986) Peptide YY release by fatty acids is sufficient to inhibit gastric emptying in dogs. Gastroenterology *91:* 1386–1389.

Perney, T. M., L. D. Hirning, S. E. Leeman, and R. J. Miller (1986) Multiple calcium channels mediate transmitter release from peripheral neurons. Proc. Natl. Acad. Sci. USA *83:* 6656–6659.

Rane, S. G., and K. Dunlap (1986) Kinase C activator 1,2-oleylacetylglycerol attenuates voltage dependent calcium current in sensory neurons. Proc. Natl. Acad. Sci. USA *83:* 184–188.

Saria, A., and E. Beubler (1985) Neuropeptide Y (NPY) and peptide YY (PYY) inhibit prostaglandin E₂ induced intestinal fluid and electrolyte secretion in the rat jejunum *in vivo.* Eur. J. Pharmacol. *119:* 47–52.

Serfozo, P., T. Bartfai, and E. S. Vizi (1986) Presynaptic effects of neuropeptide Y on [³H]-noradrenaline and [³H]-acetylcholine release. Reg. Peptide *16:* 117–123.

Stanley, B. G., S. E. Kyrkouli, S. Lampert, and S. F. Leibowitz (1986) Neuropeptide Y chronically injected into the hypothalamus: A powerful neurochemical inducer of hyperphagia and obesity. Peptides *7:* 1189–1192.

Tatemoto, K. (1982a) Neuropeptide Y: Complete amino acid sequence of the brain peptide. Proc. Natl. Acad. Sci. USA *79:* 5485–5489.

Tatemoto, K. (1982b) Isolation and characterization of peptide YY (PYY), a candidate gut hormone that inhibits pancreatic exocrine secretion. Proc. Natl. Acad. Sci. USA *79:* 2514–2518.

Tatemoto, K., M. Carlquist, and V. Mutt (1982) Neuropeptide Y: A novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature *296:* 659–660.

Thayer, S. A., S. N. Murphy, and R. J. Miller (1986) Widespread distribution of dihydropyridine sensitive calcium channels in the central nervous system. Mol. Pharmacol. *30:* 305–309.

Thayer, S. A., L. D. Hirning, and R. J. Miller (1987) Distribution of multiple types of Ca²⁺ channels in rat sympathetic neurons *in vitro.* Mol. Pharmacol. *32:* 579–586.

Unden, A., and T. Bartfai (1984) Regulation of neuropeptide Y (NPY) binding by guanine nucleotides in the rat cerebral cortex. FEBS Lett. *177:* 125–127.

Wahlestedt, C., and R. Hakanson (1986) Effects of neuropeptide Y (NPY) at the sympathetic neuroeffector junction. Can pre- and post-synaptic receptors be distinguished? Med. Biol. *64:* 85–88.

Wahlestedt, C., N. Yanaihara, and R. Hakanson (1985) Evidence for different pre- and post-junctional receptors for neuropeptide Y and related peptides. Reg. Peptides *13:* 317–328.

Walker, M. W., and R. J. Miller (1987) Neuropeptide Y receptors

coupled to G-proteins: Biochemical and physiological evidence. Soc. Neurosci. Abstr. *13:* 61.11.

Wang, Y. N., J. K. MacDonald, and R. J. Wyatt (1987) Immunocytochemical localization of neuropeptide Y like immunoreactivity in adrenergic and non-adrenergic neurons of the rat gastrointestinal tract. Peptides *8:* 145–151.

Werz, M. A., and R. L. MacDonald (1984) Dynorphin reduces calcium dependent action potential duration by decreasing voltage dependent calcium conductance. Neurosci. Lett. *46:* 185–190.

Westlind-Danielsson, A., A. Unden, J. Abens, S. Andell, and T. Bartfai

(1987) Neuropeptide Y receptors and the inhibition of adenylate cyclase in the human frontal and temporal cortex. Neurosci. Lett. *74:* 237–242.

Yamaoka, K., Y. Tanigawara, T. Nakagawa, and T. Uno (1981) A pharmacokinetic analysis program (multi) for microcomputers. J. Pharm. Dyn. *4:* 879–885.

Zukowska-Grojec, Z., S. M. Haas, and M. A. Bayarh (1986) Neuropeptide Y and peptide YY mediate non-adrenergic vasoconstriction and modulate sympathetic responses in rats. Reg. Peptides *15:* 99–110.